Oligonucleotides and Analogues

A Practical Approach

Edited by

F. ECKSTEIN

Max-Planck-Institut für Experimentelle Medizin, Göttingen, Germany

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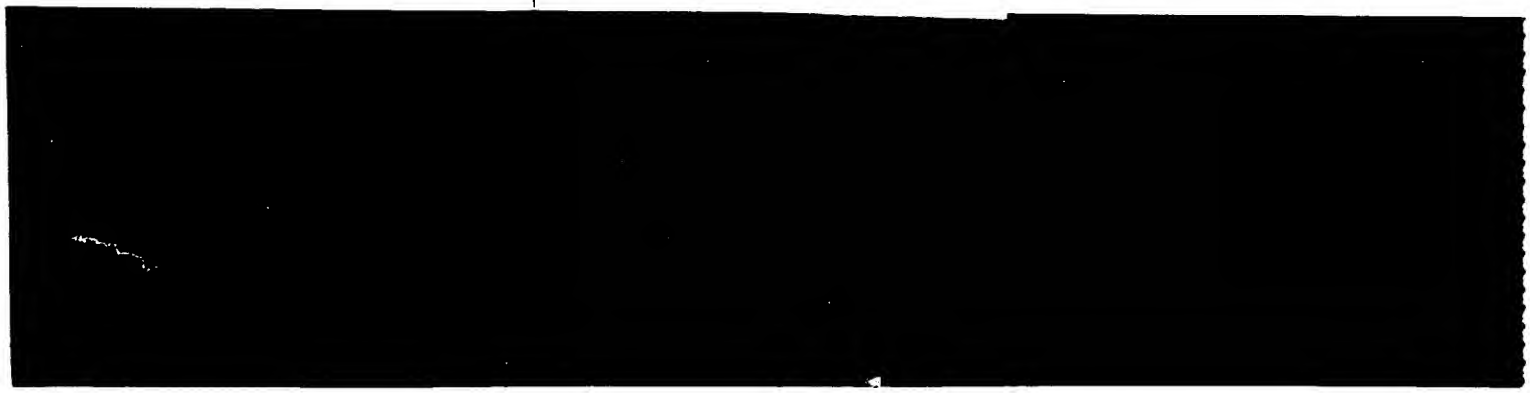

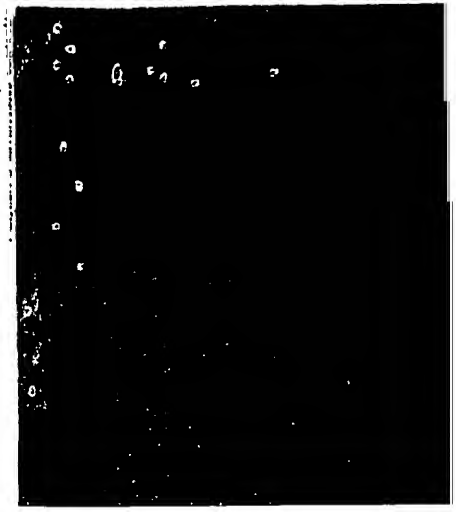

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Preface

THE chemical synthesis of oligodeoxynucleotid
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The reader will find that most of the areas
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ated rapid publication. I thank them all for

8

Oligonucleotides with reporter groups attached to the 5′-terminus

NANDA D. SINHA and STEVE STRIEPEKE

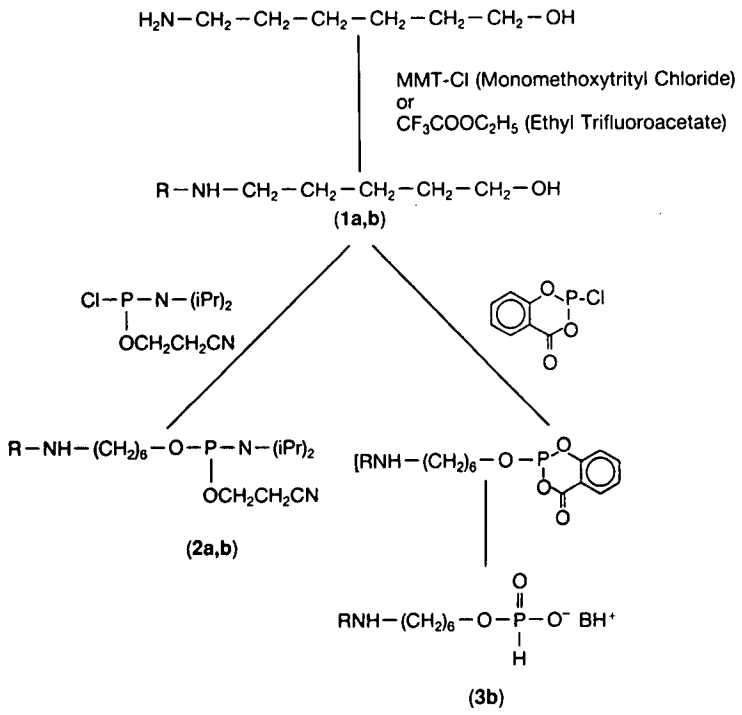
1. Introduction

Traditionally, oligonucleotides or DNA have been labelled and detected via use of ^{32}P-labelled ATP and enzymatic phosphorylation. Although of high sensitivity, this technique has intrinsic hazards, expense, and problems associated with the short half-life of this isotope. Recent advances have shown that high sensitivity detection can be achieved by alternative markers based on fluorescent, chromophoric, or chemiluminescent detection. Incorporation of these labels can be achieved by enzymatic (1–8) or chemical (9–23) methods. One of the simplest and most useful methods involves introduction of primary amino or sulphyrdryl (thiol) groups to the 5′-terminus of the chemically assembled support-bound oligonucleotide (14–23). Subsequently fluorophores, chromophores, biotin, or alkaline phosphatase may be added. Further examples are discussed in Chapter 10. With this approach the position of the amino or thiol group is defined and unambiguous, and there is negligible interference in subsequent hybridizations. Additionally, the 3′-terminus of the oligonucleotide chain is free for enzymatic manipulations as required in Sanger's sequencing and PCR amplification applications (14, 24–26). The attachment of other groups to the 5′-terminus of oligonucleotides is described in Chapter 12.

A key advantage of the 5′-terminal modification approach is that it is based on the widely adopted method of solid phase synthesis (27) and can be performed routinely on a variety of commercially available DNA synthesizers. The 5′-modification reagents are either modified nucleosides (14, 19, 20) or derived from non-nucleoside molecules (15–18, 22–23). A lengthy spacer arm is desirable to separate the incorporated functionality from the nucleic acid sequence, especially when subsequent enzyme reactions are involved. For this reason, and because simple synthesis procedures can be used in their preparation, non-nucleoside reagents have gained general acceptance.

Selection, synthesis, and application of non-nucleosidic reagents

most frequently used linkers for 5′-terminal modifications of oligo-eotides are aminohexyl and thiol-hexyl derivatives based on phos-:amidite (15–18) or H-phosphonate chemistry (22–23) (see *Figures 1* and After introduction on to oligonucleotide chains, primary amino groups react with either iso-thiocyanate or N-hydroxysuccinimide ester vatives of fluorophores, chromophores, or biotin. Similarly, free thiol ips can react with either maleimide, iodo-, or bromo-functionalized vatives of fluorophores, chromophores, or proteins with free sulphydryl ips. Furthermore, thiol-linked oligonucleotides can also be immobilized :o solid supports, (e.g. CPG-SH; Pierce) via disulphide bond (28) or :imide linkages.



a;R=CF_3CO b;R=MMT B=DBU (Diazabicycloundecene)

re 1. Preparation of protected aminohexyl phosphoramidites and H-phosphonate s as amino linkers.

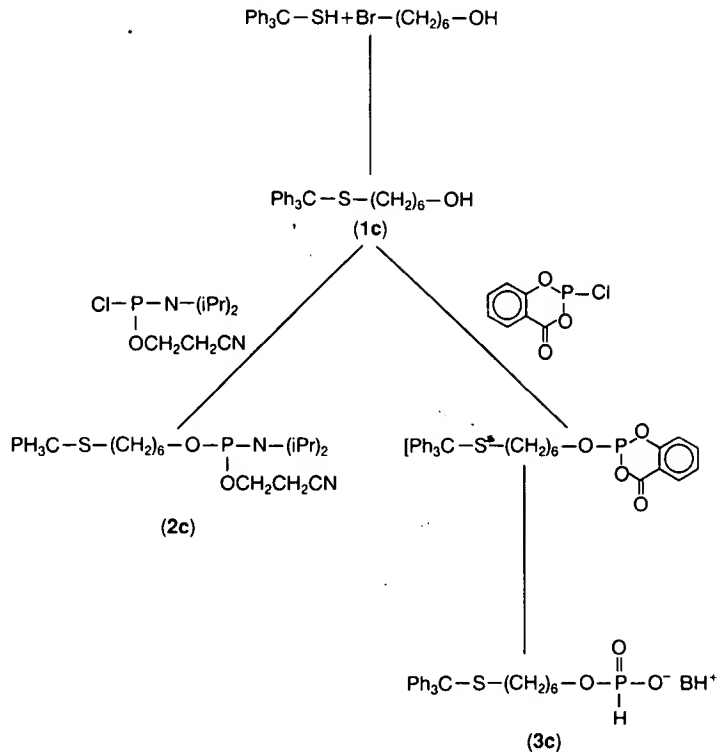
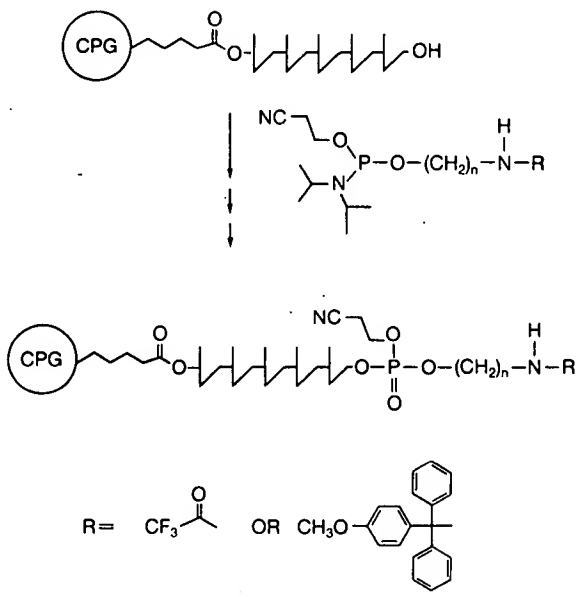


Figure 2. Preparation of protected mercapto or thio-hexyl phosphoramidites and H-phosphonate salts as thiol linkers.

The most commonly used amino-linkers for 5′-end modification are N-trifluoroacetyl (N-TFA) and N-monomethoxytrityl (N-MMT)-6-aminohexyl phosphoramidites (*Figures 1* and *2*). Standard tetrazole activation and coupling in acetonitrile incorporates these on to oligonucleotides (*Figure 3*). The removal of amino-protecting groups (TFA or MMT) differs: the trifluoroacetyl group is removed with ammonia during the final deprotection step, whereas the monomethoxytrityl group is removed by subsequent acid treatment.

Although both linkers introduce aminohexyl groups at the 5′-terminus of oligonucleotides, the monomethoxytrityl–aminohexyl linker provides advantages for purifying the amino-linked oligonucleotide from short and non-amino linked oligonucleotide using reversed phase HPLC or Oligo Pak column techniques. A MMT–amino-linked oligonucleotide can be labelled with a non-radioactive marker while still attached to the support.



gure 3. Coupling of amine-linker onto oligonucleotide chain attached to a solid pport.

The incorporation of a thiol group is achieved with S-trityl-6-mercaptohexyl erivatives (15, 22). The trityl group also provides advantages for purification. s presence facilitates the isolation of the desired product free from failure quences, short sequences, and protecting groups by reversed phase HPLC Oligo Pak column chromatography. Finally, the trityl group is removed by action with silver nitrate and the product is isolated by treatment with a TT solution.

Amino- and thiol-linked oligonucleotides both have very similar application. he choice of modification, i.e. creation of a reactive free amino or thiol, epends on the particular application. Active esters or isothiocyanate erivatives are commonly used for tagging free amino-modified oligo- ucleotides. Maleimide, bromide, iodide, or sulphonyl derivatives are itable for tagging thiol-linked oligonucleotides.

Recently, phosphoramidite derivatives of biotin (30–32) and fluorescein 3) have been introduced for incorporating these markers on to oligo- ucleotides on a solid support.

The first part of this chapter deals with the syntheses of various linkers, the cond part with the methods used to incorporate these linkers, and the final art describes tagging with non-radioactive markers.

3. Synthesis and purification of linkers†

Protocol 1. Synthesis and purification of N-trifluoroacetyl-6-aminohexanol (*Figure 1* (**1a**))

A. *Synthesis*

1. Place 6-aminohexanol (23.4 g., 200 mmol) in a clean and dry round-bottomed flask (250 ml). Dissolve the solid in chloroform (25.0 ml).
2. Add dropwise under stirring a solution of ethyl trifluoroacetate (28.4 g, 200 mmol) in chloroform (25 ml) to a solution of 6-aminohexane under an inert atmosphere. Continue stirring for another 1 h. Monitor the reaction by TLC using ethyl acetate as solvent. The reaction is generally complete by this time. The protected aminohexal has a higher R_f (0.3) than aminohexanol and can be visualized by shortwave UV-light.
3. Evaporate the reaction mixture on a rotary evaporator to a viscous oil.

B. *Purification*

1. Pack a column (5 cm diameter or an appropriate size) with Merck silica gel-60 (silica to product ratio 15:1) in 4% methanol in methylene chloride.
2. Dissolve the viscous oil in a minimum volume of the above solvent and load the solution on to the column. Elute the column with 6% methanol in methylene chloride and collect 150 ml fractions.
3. Check fractions by TLC (ethyl acetate as solvent) and visualize by shortware UV light. When elution of the product is complete, combine all product-containing fractions.
4. Concentrate the combined fractions on a rotary evaporator and finally under high vacuum to give a solid (m.p. 45–46 °C), yield is typically 50–60%. ^{1}H NMR ($CDCl_3$): δ 6.5 (S, NH, 1H); 3.6 (m, O-CH_2, 2H); 3.3 (m, N-CH_2, 2H); 1.5 (m, 2 CH_2, 4H), and 1.35 (m, 2CH_2, 4H).

Protocol 2. Synthesis and purification of N-MMT-6-aminohexanol (*Figure 1* (**1b**))

A. *Synthesis*

1. Dry 6-aminohexanol (17.5 g, 150 mmol) by co-evaporation with pyridine (2 × 100 ml; HPLC grade) under reduced pressure. Remove residual pyridine by evacuation at 0.1 mm Hg for 2 h.

† The term linker is generally applied to the molecule which attaches a primary amine or sulphydryl group to an oligonucleotide.

Protocol 2. *Continued*

2. Dissolve the solid in methylene chloride (360 ml) and while stirring add freshly distilled diisopropylethyl amine (52.5 ml, 300 mmol).
3. To the above solution, add a solution of anisylchlorodiphenyl methane (monomethoxytritylchloride; 46.5 g, 150 mmol) in methylene chloride (150 ml) by addition through a dropping funnel under an inert atmosphere.
4. After 30 min of stirring, monitor the progress of the reaction by TLC (33% acetone in hexane as solvent). Visualize the TLC plate by shortwave UV and then by spraying with an aqueous 15% solution of sulphuric acid. (R_f of the product = 0.29). The reaction is usually complete in 1 h.
5. When the reaction is complete, wash the reaction mixture with 5% $NaHCO_3$ solution (3 × 250 ml) followed by saturated NaCl solution (250 ml). After drying the methylene chloride solution over anhydrous Na_2SO_4, evaporate the solvent on a rotary evaporator to a yellow oil.

B. *Purification*

1. Pack an appropriately sized glass column with 800 g of silica gel-60 using a mixture of methanol: ethyl acetate: methylene chloride: (0.5:6:93.5 v/v/v) containing 1% pyridine.
2. Dissolve the yellow oil in 100 ml of the above solvent mixture and load the solution on to the column. Elute with a mixture of methanol:ethyl acetate:dichloromethane (1:12:87 v/v/v) and collect 200 ml fractions.
3. Check each fraction for N–MMT–aminohexanol by TLC (33% acetone in hexane, R_f = 0.29) and pool all product-containing fractions.
4. Evaporate the pooled fractions on a rotary evaporator and finally dry the residue to constant weight on high vacuum. Usually the yield of purified material is between 65 and 70%. ^{1}H NMR ($CDCl_3$): δ 8.54 (s, NH, 1H); 7.4, 7.3, 7.2, 7.15, and 6.8 (14 aromatic H); 3.8 (s, OCH_3, 3H); 3.55 (t_1 O-CH_2-2H); 2.1 (t, N-CH_2-2H); 1.45 (m, -CH_2CH_2, 4H) and 1.22 (m, -CH_2CH_2, 4H).

Protocol 3. Synthesis of S-trityl-6-mercaptohexanol (*Figure 2* (**1c**))

1. Place triphenylmethyl mercaptan (13.8 g, 50 mmol) in a round-bottomed flask (100 ml) and add ethanol (20 ml) followed by aqueous sodium hydroxide solution (2.2 g, 55 mmol in 12.5 ml water).
2. To this light pink solution, add 6-bromo-hexanol (7.6 ml = 9.1 g, 55 mmol) dropwise over 30 min. Continue stirring for an additional 1 h.

Protocol 3. *Continued*

Monitor the progress of the reaction by TLC (R_f = 0.35 in 33% acetone in hexane). The reaction is usually complete in 1–1.5 h.

3. Cool the reaction mixture in an ice-bath for 15 min and filter the reaction mixture through celite. Wash the residue with ethanol (2 × 15 ml). Combine the filtrate and washings and concentrate them under reduced pressure to a viscous oil.
4. Dissolve this viscous oil in methylene chloride (200 ml). Wash this solution with 5% aqueous sodium hydroxide (2 × 50 ml) and finally with water (2 × 100 ml).
5. Dry the methylene chloride solution with anhydrous sodium sulphate, filter, and concentrate the solution to a light yellow oil. Crystallize the product from ether–hexane (2:1 v/v) to give a colourless solid (65%), m.p. 70–72 °C. ^{1}H NMR ($CDCl_3$): δ (p.p.m.) 7.45 (m, aromatic, 6H); 7.2 (m, aromatic, 9H); 3.25 (t, -0CH_2-); 2.15 (t, S-CH_2); 1.55 (m, CH_2, 4H) and 1.35 (m, CH_2, 4H).

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4. Synthesis of the 5′-terminal modifying agents (*Figure 1*, (**2**) and (**3**))

4.1 Synthesis of phosphoramidite derivatives as modifying agents

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Protocol 4. Synthesis and purification of protected aminohexyl and thiol-hexyl phosphoramidites (*Figures 1* and *2* (**2a**), (**2b**), and (**2c**))

A. *Synthesis*

1. Dry protected hexanol [(**1a**), (**1b**), or (**1c**); 20 mmol] under high vacuum for 3 h and dissolve in freshly distilled THF (from sodium metal and benzophenone, 50 ml). Add diisopropyl ethyl amine (8.7 ml, 50 mmol) and stir at 0 °C for 10 min.
2. Add 'monochloridite'[a] (monochloro-β-cyanoethyl-N,N-diisopropylamino phosphoramidite, 5.5 ml, ~5.92 g, 25.0 mmol) dropwise through a syringe (27). The amine hydrochloride should precipitate within 5 min of addition. The reaction should be carried out under an argon atmosphere.
3. Stir for 30 min at 0 °C and allow to stir at room temp. for another 30 min to 1 h. Monitor the progress of reaction by TLC (50% ethyl acetate–hexane).

Protocol 4. *Continued*

4. When the reaction is complete, remove amine hydrochloride by filtering through a sintered glass funnel under argon and wash the solid with dry THF (2 × 20 ml).
5. Evaporate the combined filtrate to a viscous oil on a rotary evaporator. Release vacuum with argon to minimize the exposure to air.
6. Dissolve viscous oil in argon-purged ethyl acetate and wash the solution with ice-cold 5% aqueous sodium bicarbonate (2 × 50 ml) followed by saturated sodium chloride (50 ml).[b]
7. Dry the ethyl acetate solution over anhydrous sodium sulphate, filter, and concentrate the filtrate to a light yellow oil on a rotary evaporator.

[a] 'Monochloridite' is a pyrophoric compound. This reaction is only to be performed by personnel trained in handling hazardous chemicals.
[b] Sometimes aqueous work-up forms an emulsion; addition of solid sodium chloride removes this emulsion.

B. *Purification*

1. Pack a column (5 cm diameter or an appropriate size) with silica gel-60 (70–230 mesh) using 25% ethyl acetate in hexane containing 5% pyridine. The amount of silica gel should be 20 times the weight of the material to be purified.
2. Wash the silica packed column with one column volume of 25% ethyl acetate in hexane.
3. Load the sample dissolved in minimum volume of 50% ethyl acetate in hexane.

Protocol 5. Elution of protected aminohexyl and thiol-hexyl phosphoramidites

A. *N-TFA-aminohexyl phosphoramidite* (**2a**)

1. Elute the column loaded with the crude N-TFA-aminohexyl phosphoramidite with 30% ethyl acetate in hexane and collect 150 ml fractions in a screw-capped bottle or flask under argon. The product starts eluting in fraction 3.
2. Check the collected fractions by TLC (50% ethyl acetate–hexane). Detect the product by spraying with 5% $AgNO_3$ solution and heating. A grey to brown spot indicates the presence of the desired phosphoramidite (R_f = 0.5). Combine the product-containing fractions.
3. Evaporate the solvent under reduced pressure and final traces of solvent using a high vacuum pump. Check purity of the material by ^1H and ^{31}P

Protocol 5. *Continued*

NMR. Product purity is about 95–98% according to ^{31}P NMR. ^1H NMR (CD_3CN): δ 7.04 (b.s. NH, 1H); 3.8 (m, N-CH, 2H); 3.6 (m, $-CH_2$-OP, 4H); 3.35 (q, N-CH_2, 2H); 2.65 (t, $-CH_2$-CN, 2H); 1.6 (m, $-CH_2$-, 4H); ^{31}P NMR (CD_3CN) δ 144.5.

B. *N-MMT-6-aminohexyl phosphoramidite* (**2b**)

1. Elute the column loaded with the crude N-MMT-6-aminohexyl phosphoramidite (described in *Protocol 4* with 25% ethyl acetate in hexane. Collect 150 ml fractions. Fractions should be collected in a screw-capped bottle or flask under argon so that exposure to air can be avoided. The desired product starts eluting after the 2nd or 3rd fraction.
2. Check collected fractions by TLC (25% ethyl acetate in hexane). Visualize either with shortwave UV or acid spray. R_f value of the desired product is 0.38.
3. When elution is complete, combine the desired fractions and concentrate under reduced pressure using a rotary evaporator with controlled vacuum. Finally remove traces of solvents by evacuating under high vacuum to a constant weight. The yield of pure material as a viscous oil is about 70–75%.
4. Check the purity of the material by TLC, ^1H and ^{31}P NMR analysis: ^1H NMR (CD_3CN): δ 8.6 (d, NH, 1H); 7.5, 7.4, 7.2 and 6.8 (14 aromatic protons); 3.8 (m, N-CH, 2H); 3.75 (s, CH_3, 3H); 3.6 (m, $-OCH_2$-, 4H); 2.6 (t, CH_2-CN, 2H); 2.1 (q, N-CH_2, 2H); 1.65 (m, CH_2-4H); 1.35 (m, CH_2, 4H), and 1.15 (d,d, CH_3, 12H). ^{31}P NMR (CD_3CN) δ 147.95.

C. *S-trityl-6-mercaptohexyl phosphoramidite* (**2c**)

1. Elute the column loaded with the crude S-trityl-6-mercaptohexyl phosphoramidite with 30% ethyl acetate in hexane. Collect 150 ml fractions and monitor each fraction by TLC (acetone/hexane (1:2, v/v) and visualizing either with shortwave UV or acid spray). Combine pure fractions and evaporate down to a light yellow liquid.
2. Finally, remove traces of solvent by evacuating overnight under high vacuum (0.1 mm Hg).
3. Check the purity by TLC, ^1H and ^{31}P NMR. The product is usually 95% pure by ^1H and ^{31}P NMR. ^1H NMR (CD_3CN): δ 7.45 (m, aromatic 6H); 7.2 (m, aromatic 9H); 3.8 (m, N-CH, 2H); 3.6 (m, $-OCH_2$-4H); 2.6 (t, $-CH_2$-CN, 2H); 2.15 (t, S-CH_2, 2H); 1.55 (m, CH_2, 4H); 1.35 (m, CH_2, 4H), and 1.15 (d,d CH_3, 12H). ^{31}P NMR (CD_3CN): δ 148.07

4.2 Synthesis of H-phosphonate linker (*Figures 1* and *2*: (3b) and (3c))

Protocol 6. Synthesis of H-phosphonate salts of protected N-MMT-6-aminohexanol or S-trityl-6-mercaptohexanol [(**3b**) and (**3c**)]

A. *Synthesis*

1. Dry N-MMT-6-aminohexanol or S-trityl-6-mercaptohexanol (20 mmol) by evacuating under high vacuum (0.1 mm of Hg) for 6 h. Dissolve the dried alcohol in dry THF (50 ml), add diisopropylethyl amine (8.7 ml, 50 mmol). Cool this mixture in an ice-bath under argon or nitrogen with stirring.
2. To this mixture, add dropwise a solution of phosphonating reagent (22, 29) (2-chloro-5,6-benzo-1,3,2-phosphorin-4-one, 5.06 g, 25 mmol) in dry THF (25 ml) over 15 min.
3. Remove ice-bath after addition is complete and allow to stir for an additional 1 h at room temp. Usually the reaction is complete at this time which can be monitored by TLC using 10% methanol in methylene chloride. The desired product has an R_f value lower than that of the starting material.
4. When the reaction is complete, filter the insoluble amine hydrochloride and wash the solid with THF (2 × 10 ml). Combine the filtrate and washings and cool to 0 °C. Add water (5 ml) to this reaction mixture and stir for 10 min at 0 °C.
5. Evaporate the solvent on a rotary evaporator using a water aspirator. Dissolve the viscous oil in methylene chloride. Wash this solution with triethylammonium bicarbonate solution (2 × 150 ml; 0.1 M, pH 7.5) followed by water (1 × 100 ml).
6. Dry the methylene chloride solution over anhydrous sodium sulphate. Filter and concentrate the solution on a rotary evaporator to obtain a light-yellow viscous oil. The desired 'H-phosphonate linker' can be obtained by chromatographing this oil on a silica gel column.

B. *Purification*

1. Pack a column (5 cm diameter or an appropriate size) with silica gel-60 (70–230 mesh) in 5% methanol in methylene chloride containing 1% triethylamine. Amount of silica gel should be fifteen times the mass of crude material.
2. Dissolve the crude material in a minimum volume of 10% methanol in methylene chloride (25–30 ml). Apply this on to the silica gel column.

Protocol 6. *Continued*

Elute the column with 10% methanol in methylene chloride containing 0.1% triethylamine.

3. Collect 150 ml fractions and check fractions by TLC (10% methanol in methylene chloride). Impurities and starting material elute before the desired material.
4. Once impurities are removed, elute the product with 15% methanol in methylene chloride. Combine all product-containing fractions.

C. *Further procedure for N-MMT-6-aminohexyl-O-H-phosphonate linker*

1. Wash the pooled fractions (obtained from step 4 of the purification procedure) with aqueous DBU bicarbonate solution (2 × 150 ml; 0.1 M, pH 7.5) followed by water (1 × 100 ml).
2. Dry this solution over anhydrous sodium sulphate, filter, and concentrate the filtrate to a clear viscous oil.
3. Finally, remove traces of solvent under high vacuum to obtain a white foam (65% yield, >95% pure). Characterize the product by ^{31}P NMR. ($CDCl_3$: δ 2.25.

D. *Further procedure for S-trityl-6-mercaptohexyl-O-H-phosphonate linker*

1. Wash the pooled fractions (obtained from step 4 of the purification procedure) with 0.1 M triethylammonium bicarbonate (2 × 150 ml).
2. Dry the organic phase over sodium sulphate, filter, and concentrate the filtrate to a viscous oil.
3. Finally, pump it down to a constant weight (70% yield, 95% purity). Characterize the product by ^{31}P NMR: ($CDCl_3$: δ 2.42 p.p.m.

5. Incorporation of linkers on to oligonucleotide chains and purification

5.1 Synthesis of 5′-end modified oligonucleotides using phosphoramidite chemistry

Protocol 7. Incorporation of phosphoramidite linkers on an automated DNA synthesizer (*Figures 3* and *4*)

1. Dissolve amino-linkers (**2a**), (**2b**), or (**2c**) in dry acetonitrile (100–250 mg in 2–5 ml) under an argon atmosphere.

Protocol 7. *Continued*

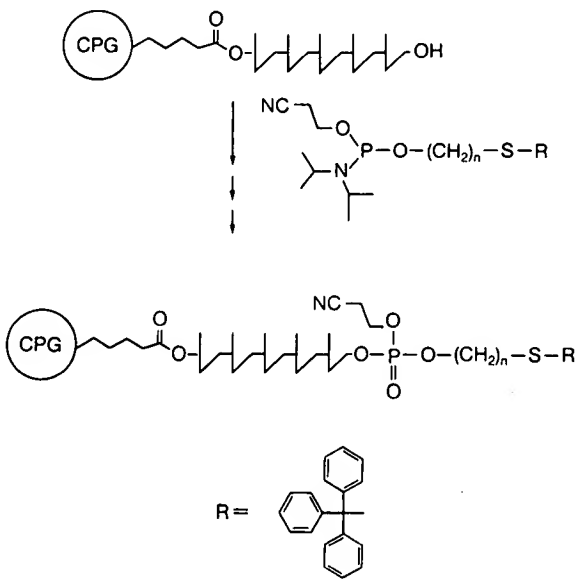

Figure 4. Coupling of thiol-linker onto an oligonucleotide chain attached to a solid support.

2. Place this solution into a clean extra reservoir (U-reservoir of the MilliGen Biosearch DNA synthesizer). Prime the line manually for a few seconds or use the priming program so that the delivery tube is filled with this reagent.
3. Write the desired sequence; the 5′-end having a base 'U' so that the modifying reagent is incorporated at the last step of synthesis by the instrument.
4. Print and verify the sequence.
5. Have 'DMT-on' option for synthesis program.
6. Start the synthesis using an appropriate scale (0.25 or 1.0 μmol) coupling program on the instrument.
7. At the end of synthesis, detach the column from the instrument and wash the support with methanol (3 × 5 ml) using a syringe to remove residual acid (from detritylation steps) and iodine (from oxidation steps).

   Complete removal of iodine is essential for thiol-linked oligonucleotides.

5.2 Synthesis of 5′-end modified oligonucleotides using H-phosphonate chemistry

Protocol 8. Incorporation of N-MMT-6-aminohexyl-O-H-phosphonate (**3b**) in an oligonucleotide on an automated synthesizer

1. Dissolve DBU salts of protected aminohexyl-O-H-phosphonate (**3b**) 50 mg/ml) in a mixture of anhydrous pyridine–acetonitrile (1:1 v/v) or commercially available H-phosphonate diluent.
2. Place this solution (3–4 ml) in an extra reservoir.
3. Rinse the line manually or using the priming program.
4. Write the desired sequence with the 5′-end bearing a residue 'U' on the instrument.
5. Print and verify the sequence so that linker is attached at the 5′-end.
6. Have 'DMT-on' option for synthesis program.
7. Start the synthesis using an appropriate synthesis scale coupling program.

Protocol 9. Incorporation of S-trityl-6-mercaptohexyl-O-H-phosphonate (**3c**) in an oligonucleotide

1. Dissolve the thio-linker (S-trityl-6-mercaptohexyl-O-H-phosphonate (**3c**) 50 mg/ml) in a mixture of anhydrous pyridine–acetonitrile (1:1) or commercially available H-phosphonate diluent.
2. Place this solution (3–4 ml) in an extra reservoir.
3. Rinse the line manually or using the prime program.
4. Write the desired sequence of oligonucleotide without linker at the 5′-end.
5. Start synthesis with 'DMT-off' option, using appropriate scale coupling program.
6. After chain assembly and oxidation with iodine solution, remove the synthesis column from the instrument.
7. Wash the support with acetonitrile–pyridine mixture to *remove the last traces of iodine.*
8. Place the synthesis column back on to the instrument.
9. Write a dimer synthesis, 'U' as the 5′-end base on the instrument.
10. Use a program which does not include oxidation with iodine or a program which has an option to stop the synthesis before oxidation.
11. When addition or coupling of linker from 'U'-reservoir is complete, after washing with wash solvent, stop the synthesis.

Protocol 9. *Continued*

12. Remove the column from the synthesizer and dry the support.
13. Pass 10 ml suspension of 10% water in carbon tetrachloride†, triethylamine, and N-methylimidazole (9: 0.5: 0.5) through the support using two 10 ml syringes for 10 min. *This oxidation prevents disulphide bond formation, generally obtained with iodine-solution oxidation.*
14. Remove this mixture, wash with acetonitrile. Discard the washing as a halogenated waste.

† Gloves must be used while handling carbon tetrachloride, a carcinogen. Perform this oxidation step in the fume hood.

5.3 Deprotection and isolation of 5′-modified oligonucleotides from solid support

This step can be performed following the protocol recommended by the instrument manufacturers or by the steps given below.

Protocol 10. Deprotection and removal of oligonucleotides from supports

1. Transfer the dried support into a screw-capped vial or screw-capped Eppendorf tube (1.5 ml).
2. Add 300 µl (0.25 µmol scale) or 1.0 ml (1.0 µmol scale) of conc. NH_4OH solution (30%).
3. Close the cap tightly and incubate the suspension at 55 °C for at least 5 h to overnight (*a longer period should be given for G-rich sequences*).
4. Cool to 0 °C and transfer the supernatant into another microfuge tube.
5. Rinse the support with same amount of water (HPLC grade or double distilled). Add this washing to the ammonia supernatant (step 4). *This ammonia solution contains full-length oligonucleotide with either a free aminohexyl group at the 5′-end (in the case of N-TFA-aminohexyl phosphoramidite incorporated) or protected amino- or thiolhexyl-linked oligonucleotide together with non-nucleosidic material and short sequences.*

Purification of oligonucleotides with free aminohexyl linkers can be achieved by anion exchange HPLC, ethanol precipitation (*Protocol 11*) or polyacrylamide gel electrophoresis.

Protocol 11. Purification by ethanol precipitation

1. Concentrate the ammonia solution (obtained from Protocol 10, steps 4 and 5) to dryness in a SpeedVac without heating.
2. Suspend the dried material in 200 µl of water and evaporate again.

Protocol 11. *Continued*

3. Dissolve the crude material in 200 µl of 0.5 M ammonium acetate solution, add 1.0 ml 95% ethanol. Mix the solution by vortexing. Cool the mixture at −20 °C for 30 min and then centrifuge for 3 min.
4. Remove the supernatant, wash the residue with cold ethanol (−20 °C). Shorter sequences, benzamide, and isobutyramide are removed by this step.
5. Dry the residue and resuspend the material in known volume of water (200 µl).
6. Measure the amount of oligonucleotide present in the solution at 260 nm. *This material is sufficiently pure for attachment of a non-radioactive marker molecule at the 5′-terminal primary amino group.*

If absolutely pure amino-linked oligonucleotide is required, the following standard gel electrophoresis procedure should be followed. *For sequences up to 30 bases long, use 20% polyacrylamide; for those up to 50 bases long, use a 15% polyacrylamide gel containing 7.0 M urea. The mobility of an amino-linked oligonucleotide is slightly slower than that of a non-modified oligonucleotide.* Follow any standard electrophoresis protocol—this is a routine procedure in molecular biology laboratories. The gel is then visualized by UV-shadowing at 260 nm and the product band is eluted from the gel.

Purification of oligonucleotides linked with protected primary amino- or thio-linkers can be achieved by reversed phase chromatography using either an Oligo-Pak column (MilliGen/Biosearch) (*Protocols 12* and *13*) reversed phase or HPLC (C_{18} Nova Pak or Bonda Pack, Waters).

Protocol 12. Oligo-Pak column purification for amino-linked oligonucleotides. Follow the protocol recommended by the suppliers.

1. First wash the Oligo-Pak column with 3 × 5 ml acetonitrile followed by 3 × 5 ml 1.0 M triethylammonium acetate solution (pH 7.0).
2. Using a syringe, load the entire sample (0.25 µmol scale) or one-third of a 1.0 µmol scale synthesis obtained from deprotection described in *Protocol 10*.
3. Wash the column 3 times as described in step 1.
4. Save the effluent: it may contain some full-length desired product.
5. Wash the support with 3 × 5 ml of 3% aqueous NH_4OH solution (10 times diluted concentrated ammonium hydroxide).
6. Subsequently wash with 3 × 5 ml of water.
7. Treat the Oligo-Pak column support with 1 × 5 ml of 2% trifluoroacetic

Protocol 12. *Continued*

acid solution over 2 min and wash the support immediately with water (3 × 5 ml) to remove residual trifluoroacetic acid.

8. Elute the product with 20% acetonitrile in water in two 1 ml fractions. Concentrate the eluant to dryness in a SpeedVac.
9. Dissolve the dried material in HPLC-grade water (200 µl) and dry the solution again.
10. Finally, dissolve the dried material in a known volume of water. Determine the amount of oligonucleotide in this solution by measuring the absorption at 260 nm.

Protocol 13. Oligo-Pak column purification for S-trityl-thiol-linked oligonucleotides

1. Load the entire or 1/3 volume of the sample obtained from *Protocol 10* on to the equilibrated Oligo-Pak column.
2. Wash with 3% aqueous ammonium hydroxide solution (3 × 5 ml) and then with water (3 × 5 ml).
3. Elute the product as the protected thiol-linked oligonucleotide with 40% acetonitrile in water (2 × 1 ml).
4. Concentrate the eluant to dryness in a SpeedVac. Dissolve the residue in a known amount of HPLC-grade water (200 µl) and determine the amount of oligonucleotide by measuring the absorption at 260 nm. *Removal of the trityl group should be carried out just prior to its use by treatment with silver nitrate solution followed by aqueous DTT solution. Use the free sulphydryl linked oligonucleotide immediately for non-radioactive marker incorporation.*

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6. Incorporation of non-radioactive marker molecules on to free primary amine or sulphydryl-linked oligonucleotides

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Protocol 14. Solution phase labelling of 5'-end amino-linked oligonucleotides with isothiocyanate or N-hydroxysuccinimide ester derivatives of fluorescein, rhodamine, Texas Red, biotin or NBD-fluoride.

A. *Marker incorporation*

1. Dissolve partially or fully purified amino-linked oligonucleotide (20–30 A_{260} units obtained from *Protocol 11*) in 250 µl of a mixture of 1.0 M $NaHCO_3/Na_2CO_3$ (pH 9.0).

Protocol 14. *Continued*

2. Check the pH of this solution using pH paper and *make sure it is basic.*
3. Add 500 µl of a solution of the dye derivative (10–15 mg) in a mixture of 1.0 M, $NaHCO_3/Na_2CO_3$ buffer pH = 9.0; DMF: Water (5:2:3 v/v).
4. Vortex the mixture and wrap the Eppendorf tube with aluminum foil to prevent light exposure.
5. After 20 h of incubation at room temperature, concentrate the mixture to a 250 µl volume.

B. *Removal of excess marker*

1. Pack a column (20 cm × 1.0 cm) with presuspended Sephadex G-25.
2. Equilibrate the column with HPLC-grade water (30–40 ml).
3. Apply the concentrated dye or biotin-labelled reaction mixture onto the column.
4. Elute the column with water. A faintly coloured band separates from the main coloured band, which contains the dye-tagged oligonucleotide.
5. Collect 1.0 ml fractions in Eppendorf tubes. *The desired product starts eluting after the void volume. Most of the desired product is eluted in fractions 3–9. Some fractions eluting after that are colourless followed by heavily coloured solution. The latter contains the unincorporated excess dye.*
6. Check the absorption of the faintly coloured solutions at 260 nm.
7. Concentrate the fractions which contain most of the material.
8. Combine these concentrated fractions into a single Eppendorf tube. *Usually 15–25 A_{260} units (80%) is obtained, which is free from excess dye. The purity of this material is generally better than 90–95%. If necessary, the product can further be purified by electrophoresis (20% PAGE) or by HPLC.*

Protocol 15. Labelling of 5'-end thiol linked oligonucleotide with maleimide derivative of biotin, 7-N,N-diethylamino-4-methyl-3-(4'-maleimido-phenyl)-coumarin, eosin, or monobromo bimane.

A. *Removal of trityl group*

1. Place a solution of purified S-trityl-thiol-linked oligonucleotide (30 A_{260} units; 100 µl) in an Eppendorf tube.
2. Add silver nitrate solution (25 µl, 1.0 M) and vortex the mixture.

Protocol 15. *Continued*

3. After one hour, add aqueous DTT solution (50 µl, 1.0 M), vortex again and centrifuge the mixture for 15 min.
4. Place the supernatant (containing free thiol-linked oligonucleotide and excess DTT) in a separate Eppendorf tube.

B. *Removal of excess DTT*

1. Add 500 µl of ethyl acetate to the supernatant, vortex, and centrifuge.
2. Remove ethyl acetate in the upper layer. Repeat this process once more. This process removes most of the excess DTT.

C. *Incorporation of marker molecules*

1. Immediately add a solution of maleimide or bimane derivative (10–15 mg) in DMF/THF (1:1 v/v) (500 µl) to the aqueous layer.
2. Vortex the reaction mixture, wrap the tube in aluminum foil and incubate at room temp. for 20 h or overnight.
3. Concentrate the mixture to a 200 µl volume.

D. *Removal of excess markers*

1. Place this reaction mixture onto a pre-equilibrated Sephadex column as described in *Protocol 14*.
2. Elute the product with HPLC-grade water. A faintly coloured band separates from the main coloured band. *The faintly coloured band contains most of the desired product.*
3. Collect 500 µl to 1.0 ml fractions in Eppendorf tubes. Most of the product elutes at the void volume of the column, usually in fractions 3–9.
4. Check the absorption of these fractions at 260 nm.
5. Concentrate the fractions which contain most of the material.
6. Combine these fractions in a single Eppendorf tube. *Usually 20–25 A_{260} units of purified material is obtained, which may contain up to 10% unincorporated dye and non-dye linked oligonucleotide.* If necessary, the product can be further purified by reverse-phase HPLC or 20% PAGE to result in homogeneous product.

Protocol 16. Labelling of 5'-end amino-linked oligonucleotide with a non-radioactive marker on a solid support (see *Figure 5*).

A. *Introduction of amino linker*

1. Introduce N-MMT–aminohexyl linker at the 5'-end of synthetic oligonucleotide as described in *Protocol 7* or *8*.

Protocol 16. *Continued*

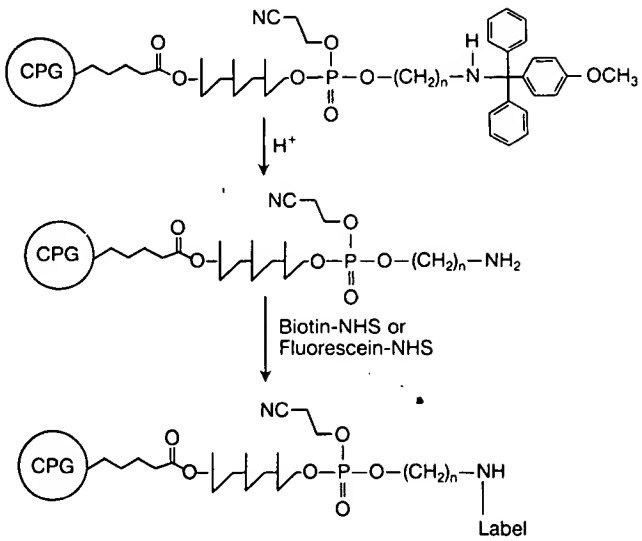

Figure 5. Introduction of non-radioactive reporter group at the 5'-end of an amine-modified oligonucleotide on a solid support. Biotin-NHS = N-hydroxysuccinicimide ester of biotin.

2. Remove MMT-group (monomethoxytrityl) from the oligonucleotide chain to generate a free amino group by passing a deblock solution until the support is free from yellow colour. *Rinse the support with wash solvent. These steps can be performed on the instrument by manual mode.*
3. Finally, remove the synthesis column and wash with a solution of 10% diisopropylethyl amine in dimethylformamide: (v/v; 2 × 5 ml). This removes the acid completely and generates a free amino group at the 5'-end of the oligonucleotide attached to the solid support.

B. *Introduction of marker molecules*

1. Incubate the supports present in the column between two 1 ml syringes with 500 µl solution of the isothiocyanate or the N-hydroxysuccinimide derivative of fluorescein, rhodamine, biotin, or ethylene diamine tetracetic acid anhydride (in 10% diisopropylethyl amine in DMF, 5 mg/ml).
2. After 1 h of incubation of the dark at room temp., wash the support with DMF to remove the excess dye using a 5 or 10 ml syringe [4 × 5 ml or 3 × 10 ml DMF washing, followed by acetonitrile (2 × 5 ml).
3. Dry the support by blowing air through with a syringe. Transfer the support into a screw cap Eppendorf tube and incubate with 1 ml of conc. NH_4OH either at 55 °C for 5 h or at room temp. for 24 h.

Protocol 16. *Continued*

4. Transfer the coloured supernatant into a separate Eppendorf tube and rinse the support with 2 × 250 µl of water. Combine the washings.
5. This solution contains a mixture of dye or non-radioactive marker tagged oligonucleotide, short DNA sequences, protecting groups and a small amount of free marker molecules (see *Figure 6*).

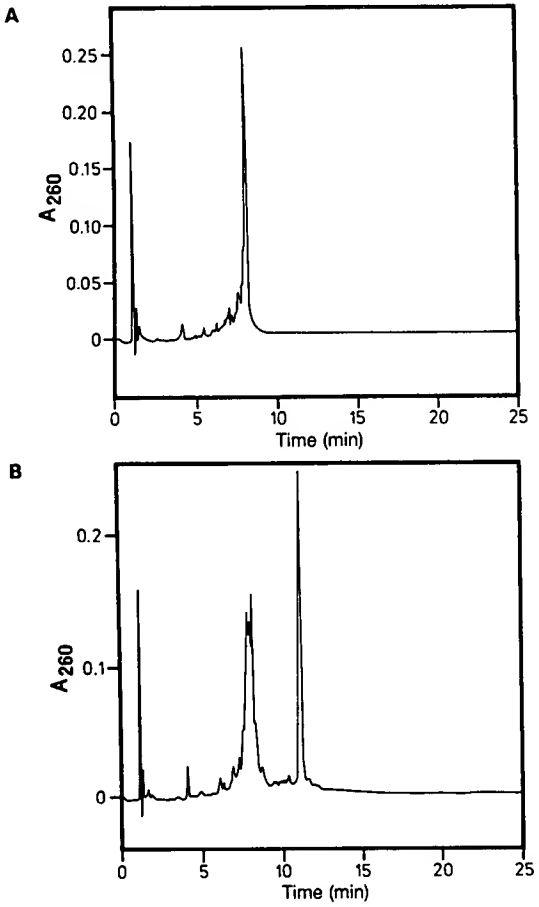

Figure 6. Reversed phased HPLC analysis of unpurified synthetic oligonucleotides. (**A**) Non-modified oligonucleotide (24mer) and (**B**) fluorescein isothiocyante attached to an amino-linked oligonucleotide (24mer) (peaks I = unreacted oligonucleotide and II = fluorescein-tagged oligonucleotide) on solid support. HPLC conditions: Column Waters C_{18} Delta Pak, 5 µm spherical particle; buffers: A = 0.1 M TEAA pH = 7.0, B = 100% acetonitrile; Gradient: linear gradient 0–1.0 min 95% A and 5% B then 1.0–25.0 min 40% B, Detection: 260 nm.

Protocol 16. *Continued*

C. *Purification by Oligo-Pak by a modified procedure*

1. Equilibrate Oligo-Pak column with acetonitrile (2 × 5 ml), water (2 × 5 ml) and 1.0 M triethylammonium acetate, pH 7.0 (2 × 5 ml).
2. Load the reaction mixture (1:1 diluted with water) on the column with a syringe in 3 steps.
3. Wash the column with 3% NH_4OH (2 × 5 ml) followed by water (2 × 5 ml).
4. Wash with 7% acetonitrile in water (1 × 5 ml) to remove the uncoupled amino-linked oligonucleotide.
5. Elute the dye-tagged oligonucleotide with 20% acetonitrile in two 1 ml fractions.
   Combine these fractions, lyophilize solution and check the purity of the product by HPLC (see *Figure 7*) or gel electrophoresis.

7. Applications of oligonucleotides carrying non-radioactive reporter molecules

7.1 DNA sequencing

Fluorescein, rhodamine, or other fluorophores linked to the 5′-end of oligonucleotides have been used as chain extension primers in automated DNA sequencing (14, 24–33). Recently, biotin-linked oligonucleotides have also been used in DNA sequencing followed by chemiluminescent (25) and colourimetric (26) detections.

7.2 Diagnostic probes

With the improvement of non-radioactive detection systems, the use of synthetic oligonucleotides with non-radioactive reporter groups as diagnostic probes is increasing. Examples are:

(a) A colourimetric method for visualizing biotin-labelled DNA probes hybridized to DNA or RNA immobilized on nitrocellulose (1b).

(b) A colourimetric method for DNA hybridization (34).

(c) Chemically modified nucleic acids as immuno-detectable probes in hybridization experiments (35)

(d) Comparison of non-radioisotopic hybridization assay methods using fluorescent, chemiluminescent, or enzyme-labelled synthetic oligonucleotides (36).

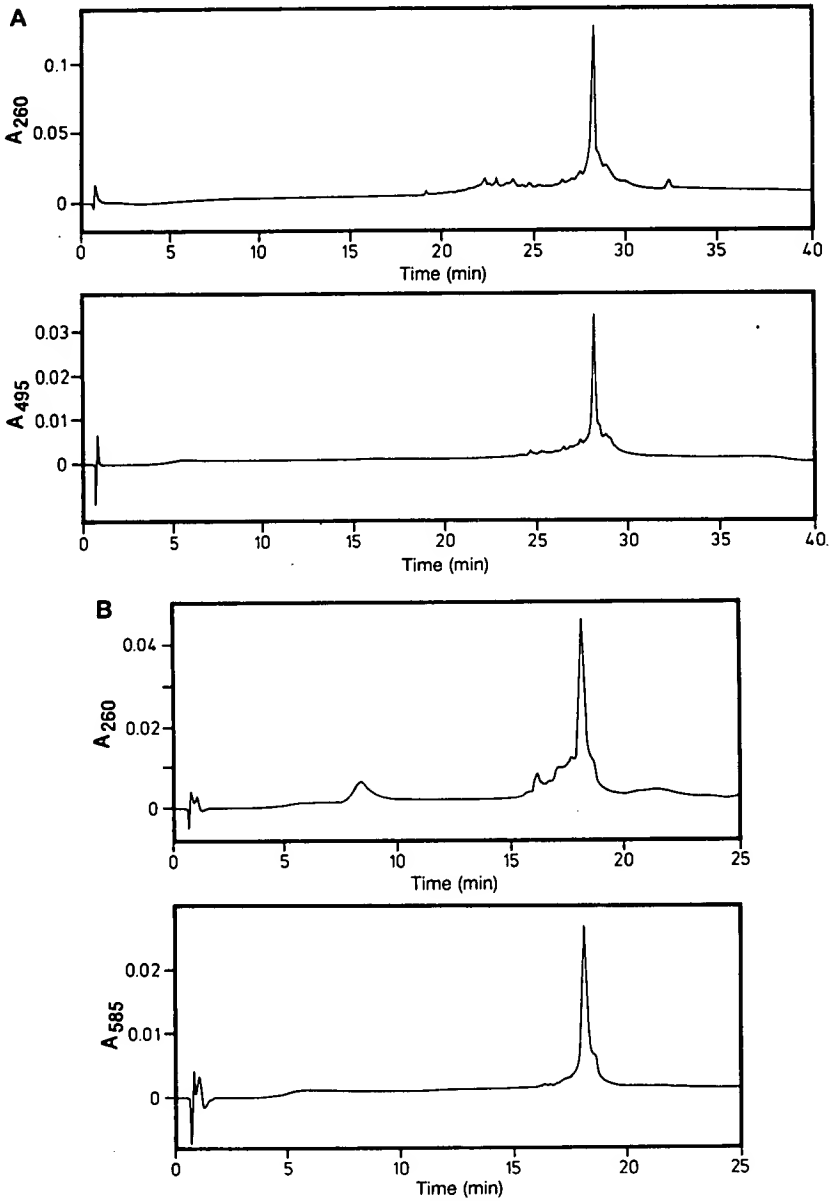

Figure 7. Anion exchange HPLC analysis of Oligo-Pak column-purified, fluorescein isothiocyanate-attached oligonucleotide (24mer) (**A**) and rhodamine isothiocyanate-attached oligonucleotide (15mer) (**B**). HPLC conditions: column: Water Gen-Pak Fax anion-exchange; buffers: A = 25 mM Tris–HCl in water pH = 7.5, B = 25 mM Tris–HCl/1.0 M NaCl in water pH = 7.5; gradient: linear gradient 0–1.0 min 100% A, then 1–35 min 0–60% B; detection: (A) at 260 and 495 nm, (B) at 260 and 585 nm.

(e) Rapid chemiluminescent nucleic acid assays for the detection of TEM-1 β-lactamase-mediated penicillin resistance in *Neisseria gonorrhoea* and other bacteria (37).

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Appendix: Chemical suppliers

Protection of 6-aminohexanol

ethyl trifluoroacetate	Aldrich
6-aminohexanol	Aldrich
p-methoxyphenyldiphenylmethyl chloride (MMT-chloride)	Aldrich
N,N-diisopropylethyl amine	Aldrich
silica gel-60 (70–230 mesh)	Merck
precoated silica gel GF-254 plates	Merck
sodium sulphate	Merck

Protection of 6-mercaptohexanol

triphenylmethyl mercaptan	Aldrich
6-bromo-1-hexanol	Sigma
sodium hydroxide	Merck
sodium sulphate	Merck

Synthesis of amino- or thio-hexyl phosphoramidite linkers

monochloro-β-cyanoethoxy-N,N-diisopropyl-amino phosphine (phosphitylating agent)	Applied Biosystems Biosyntech American Biometrics Cruachem Glen Research
phosphoramidite linkers now also available from	Milligen-Biosearch Clonetech Glen Research

Synthesis of amino- or thio-hexyl phosphonate linkers

2-chloro-4H,1,3,2-benzodioxaphosphorin-4-on (phosphonylating agent)	Aldrich
diazobicyloundecen	Aldrich

Incorporation of linker on to oligonucleotide chain

Phosphoramidite linkers	
deoxynucleoside phosphoramidites and ancillary DNA synthesis reagents	Milligen-Biosearch Applied Biosystems American Biometrics
H-phosphonate linkers	
deoxynucleoside H-phosphonates and ancillary DNA synthesis reagents	Milligen-Biosearch Applied Biosystems American Biometrics
triethylamine	J. T. Baker Aldrich
carbon tetrachloride	Aldrich
N-methyl imidazole	Aldrich
Deprotection and purification	
concentrated ammonium hydroxide solution (30%)	Aldrich
materials for polyacrylamide gels (acrylamide, bisacrylamide, ammonium persulphate)	Aldrich
Oligo-Pak	Milligen-Biosearch
triethylamine	J. T. Baker
glacial acetic acid	Aldrich
trifluoroacetic acid	Aldrich

Reporter groups attached to the 5'-terminus

Attachment of a non-radioactive markers

Amino-linked oligonucleotides	
dimethylformamide (analytical grade; free from primary and secondary amines)	
triethylamine, isothiocyanate, or N-hydroxy-succinimide ester derivatives of fluorescein,	Molecular Probes
rhodamine and Texas Red	Aldrich
N-hydroxysuccinimide ester of biotin	Molecular Probes Pierce
Thiol-linked oligonucleotides	
Silver nitrate	Aldrich
dithiothreitol (DTT)	Sigma
maleimide derivative of biotin	Sigma
eosin	Molecular Probes
7-diethylamino-4-methyl-3-(4'-maleimidlphenyl)-coumarin	Molecular Probes
monobromo bimane	Molecular Probes

Solvents

Analytical or HPLC grade solvents are commonly used. These are dichloromethane, ethyl acetate, pyridine, N,N-dimethylformamide(DMF), tetrahydrofuran (THF), methanol, hexane, and acetonitrile, which can be obtained from any reputable supplier.

9

Site-specific attachment of labels to the DNA backbone

NANCY E. CONWAY, JACQUELINE A. FIDANZA, MARYANNE J. O'DONNELL, NICOLE D. NAREKIAN, HIROAKI OZAKI, and LARRY W. McLAUGHLIN

1. Introduction

Sequence-specific attachment of reporter groups, drug derivatives, or chemically reactive species to DNA sequences has the potential to provide new materials for detailed spectroscopic and biophysical studies as well as a host of new DNA therapeutics and diagnostics. The covalent binding of a variety of such agents at specific locations within the nucleic acid sequence can be achieved by a number of procedures depending on whether the nucleobase, carbohydrate, or phosphate residue is employed as the site of attachment. These procedures often exploit the availability of specific functional groups (such as terminal phosphomonoesters, see Chapter 8) or the reactivity of selected sites on the purine or pyrimidine building blocks (such as the C5 position of pyrimidines, see Chapter 11) in order to attach an appropriate linker or the active agent directly. While the manner in which the nucleic acid is labelled may be dictated by the specific study involved, in general the principles of simplicity and versatility are best employed to guide the choice of labelling procedure.

Some consideration should also be given to the structural effects the label or agent will have the nucleic acid. For example, the addition of an agent or label to a terminal phosphomonoester would be unlikely to alter the structure or stability of a double-stranded or even triple-stranded complex. On the other hand, internal labelling may be more advantageous if the product complex involves an interstrand covalent cross-link or an intercalating agent. Labelling of a site within a sequence has generally relied upon the synthesis of a modified nucleoside building block in which a linker is incorporated for attachment of the label. However, in some cases this can destabilize the duplex structure, for example when the exocyclic amino group of the cytosine residue was used as a site of attachment, the labelled helices exhibited biphasic melting curves (1, 2) suggesting local or even global structural